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Functional Interaction between DP-1 and p53

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The cellular transcription factor DRTF1/E2F and the tumor suppressor protein p53 play important roles in controlling early cell cycle events. DRTF1/E2F is believed to coordinate and integrate the transcription of cell cycle-regulating genes, for example, those involved in DNA synthesis, with the activity of regulatory proteins, such as the retinoblastoma tumor suppressor gene product (pRb), which modulate its transcriptional activity. In contrast, p53 is thought to monitor the integrity of chromosomal DNA and when appropriate interfere with cell cycle progression, for example, in response to DNA damage. Generic DRTF1/E2F DNA binding activity and transcriptional activation arise when members of two distinct families of proteins, such as DP-1 and E2F-1, interact as DP/E2F heterodimers. In many cell types, DP-1 is a widespread component of DRTF1/E2F DNA binding activity which when expressed at high levels oncogenically transforms embryonic fibroblasts. Here, we document an association between DP-1 and p53 and demonstrate its presence in mammalian cell extracts. In vitro p53 interacts with an immunochemically distinct form of DP-1 and in vivo can regulate transcription driven by the DP-1/E2F-1 heterodimer. At the biochemical level, p53 competes with E2F-1 for DP-1, with a consequent reduction in DNA binding activity. Mutational analysis defines within DP-1 a C-terminal region required for the interaction with p53 and within p53 an N-terminal region distinct from that required to bind to MDM2. Our results establish DRTF1/E2F as a common cellular target in growth control mediated through the activities of pRb and p53 and suggest an alternative mechanism through which p53 may regulate cellular proliferation.

The transition from G_1 into S phase is an important regulatory point in cell cycle progression, since a number of genes must be transcriptionally activated in order that cells may continue through the cell cycle. Many of these genes contain within their control sequences binding sites for the cellular transcription factor DRTF1/E2F, which is widely believed to play an important role in regulating transcription during early cell cycle progression (29).

The potential role of DRTF1/E2F in cell cycle control is underscored by the properties of the proteins which are known to influence its transcriptional activity. For example, a group of proteins which negatively regulate the cell cycle, including the retinoblastoma tumor suppressor protein (pRb) and relatives p107 and p130 (collectively known as pocket proteins), bind to and inactivate the transcriptional activity of DRTF1/E2F (5, 8, 9, 40). These interactions can be deregulated in tumor cells, for example, through the action of viral oncoproteins such as adenovirus E1a (35), and furthermore are known to be temporally influenced by cell cycle progression (9, 43). Another group of molecules which regulate cell cycle transitions, the cyclins and their catalytic regulatory subunits, interact with DRTF1/E2F (2, 10, 30, 34). Cyclins A, E, and D together with an appropriate catalytic subunit are believed to influence the activity of pocket proteins (20, 42), and direct phosphorylation by a cyclin A-cdk2 kinase reduces the DNA binding activity of DRTF1/E2F (11, 27). Overall, the nature of the target genes and the physiological properties of the afferent signalling proteins suggest that the activity of DRTF1/E2F plays a pivotal role in regulating and coordinating early cell cycle progression.

It is now known that generic DRTF1/E2F DNA binding activity defined in mammalian cell extracts results from an array of heterodimers made up from two distinct families of proteins, E2F and DP (E2F/DP heterodimers constituting physiological DRTF1/E2F). To date five members of the E2F family have been defined, E2F-1 to E2F-5, which, in the context of an E2F/DP heterodimer, dictate the interacting pocket protein (6, 7, 17, 19, 22, 25, 30, 41). Three members of the DP family are known to exist (13, 14, 37). Of these, DP-1 is the most widespread member of DRTF1/E2F yet defined (3, 4), being, for example, in all of the various forms of DRTF1/E2F which occur during the cell cycle in 3T3 cells (4).

Both E2F and DP proteins are endowed with growth-promoting activity, since in a variety of assays they have been shown to possess proto-oncogenic activity (12, 23, 24, 44, 46). For example, overexpression of DP-1 or DP-2 together with activated Ha-*ras* causes transformation of rat embryo fibroblasts, which, interestingly, is apparent in the absence of a cotransfected E2F family member (24).

In this study, we document an association between DP-1 and p53 and demonstrate its presence in mammalian cell extracts. In vitro p53 interacts with an immunochemically distinct form of DP-1 and in vivo can inactivate transcription driven by the DP-1/E2F-1 heterodimer. At the biochemical level, p53 competes with E2F-1 for DP-1 with a consequent reduction in DNA binding activity. These results establish DRTF1/E2F as a common cellular target in two distinct pathways of growth control and argue that the pathways regulated by the pRb and

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p53 tumor suppressor proteins are functionally integrated. Similar results have recently been reported by others (36).

MATERIALS AND METHODS

Antibodies and immunological methods. Monoclonal antibody 421 against p53 has been previously described (15). Anti-DP-1(A) is a rabbit polyclonal antipeptide serum raised against a peptide representing an N-terminal region in DP-1 and has been previously described (4, 14). Anti-DP-1(D) rabbit polyclonal antipeptide serum (4) and monoclonal antibody 32.3 were raised against a peptide representing a C-terminal region in DP-1; these two antisera possess very similar immunochemical properties.

Immunoblotting was performed by using standard techniques. Primary antibodies were used at 1:200 dilution for polyclonal antibodies, at 1:100 dilution for affinity-purified antibodies, and neat for monoclonal antibody supernatants. Where necessary, either homologous peptide or control peptide was added at 10 nmol/ml to diluted antibody to assess the specificity of the reaction. Secondary antibodies were either alkaline phosphatase-conjugated goat anti-rabbit (Promega) at a 1:750 dilution or rabbit anti-mouse (Dako) at 1:1,000. For immunoprecipitation, 10^7 cells were harvested in 500 μl of LSL buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Nonidet P-40, 2 μg of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride) and spun at 4°C for 10 min at 13,000 rpm. Antibody (20 μl of polyclonal, 100 μl of affinity-purified polyclonal, or 100 μl of monoclonal antibody supernatant) was added to 200 μl of lysate in the presence, where necessary, of 40 nmol of specific or nonspecific peptide and incubated for 1 h on ice. Immune complexes were collected by the addition of 150 μl of 10% (vol/vol) protein A beads (Sigma) in LSL buffer and washed extensively (at least three times) in LSL buffer. Proteins were released in sodium dodecyl sulfate (SDS) sample buffer, electrophoresed, and immunoblotted.

Gel retardation. Gel retardation was performed with 5 to 10 μg of F9 embryonal carcinoma (EC) cell extract and an E2F binding site taken from the E2a promoter as described previously (14). To supershift the DRTF1/E2F binding activity, 10 μl of monoclonal antibody 32.3 supernatant was added to the binding reaction mixture together with 2 nmol of competing or noncompeting peptide, and the mixture was incubated for 10 min at 30°C. To assess the DRTF1/E2F DNA binding activity immunoprecipitated by 32.3, immunoprecipitations were performed as described above in the presence of either homologous or unrelated peptide. Competing homologous peptide (4 nmol/μl in LSL buffer) was added after washing of the immunoprecipitate in LSL buffer, and the supernatant subsequently was assayed for DRTF1/E2F DNA binding activity.

The effect of p53 on the E2F site binding activity of the E2F-1/DP-1 heterodimer was assayed by using in vitro-transcribed and -translated DP-1 (pG4DP-1 [3]) and E2F-1 (pSP72 [25]). In vitro transcription and translation was carried out in a TNT T7/SP6 coupled reticulocyte lysate system (Promega). pH6-mmp53 wt encodes a His-tagged complete mouse p53 protein (kindly supplied by Gunnar Weidt and Wolfgang Deppert). His-tagged murine p53 was purified from a 500-ml pellet of isopropylthiogalactopyranoside (IPTG)-induced bacterial culture. The bacterial pellet was resuspended in 10 ml of denaturing buffer (100 mM sodium phosphate, 10 mM Tris base, 6.0 M guanidine hydrochloride, 30 mM imidazole [pH 8.0]) and gently stirred for 2 h at room temperature. $MgCl_2$ was added to a final concentration of 5 mM, and cellular debris were cleared by repeated centrifugation at 4°C. Four hundred microliters of nickel-agarose (solid; Qiagen) was added to the supernatant, which was then rotated for 1 h at room temperature. The resin was washed stepwise with two 50-ml volumes each of denaturing buffer (pH 8.0), denaturing buffer (pH 6.4), and renaturing buffer (25 mM sodium phosphate [pH 7.0], 300 mM NaCl, 10 mM β-mercaptoethanol) containing 1 M, then 0.1 M, and lastly no guanidine hydrochloride. Protein was eluted off the resin by sequential washes with imidazole buffer (150 mM imidazole, 100 mM NaCl, 50 mM Tris-HCl [pH 8.0]) and analyzed by SDS-polyacrylamide gel electrophoresis. An equal amount of heat-denatured or nondenatured His-tagged p53 was added to the binding reaction mixture.

Fractionation. Heparin-Sepharose and E2F binding site affinity chromatography of F9 EC cell extracts was performed as previously described (14). Fractions were assayed for DRTF1/E2F DNA binding activity by gel retardation and immunoblotted.

Binding assay for p53. His-tagged murine p53 was purified as described above. DP-1 mutants 1-238, 1-218, and 1-211 were constructed following exonuclease treatment of pG4DP-1. Mutants 205-331, 171-331, and 133-331 were made by PCR, the resulting DP-1 fragment being inserted into a pCMV vector backbone along with a synthetic translational initiation sequence. DP-1 MT was made using the Altered Sites in vitro mutagenesis system (Promega), residues A-172 and L-173 being changed to glycine residues. Finally, DP-1 73-410 and 1-341 were made by restriction enzyme digestion of full-length DP-1. Glutathione *S*-transferase (GST)–E2F-1, GST-pRb, and GST were induced and purified by conventional procedure. For the in vitro binding assay shown in Fig. 3, 15 μl (about 100 ng) of fusion protein bound to the appropriate agarose (glutathione-agarose or nickel-agarose) was incubated with F9 EC whole cell extract with constant rotation for 2 h at 4°C. The suspension was centrifuged and repeatedly washed with LSL buffer, resuspended in SDS loading buffer, and immunoblotted with anti-DP-1(A). For the in vitro binding assay shown in Fig. 4, 50 μg of His-tagged p53 was incubated with 5 μl of in vitro-transcribed and -translated DP-1, DP-1 MT, or E2F-1 in PI buffer (phosphate-buffered saline [PBS] containing 20 mM imidazole, 1 mg of bovine serum albumin [BSA] per ml, and 20% Tween 20). After 30 min at room temperature, 20 μl of nickel-agarose beads (1:3, vol/vol) was added, and the incubation time was extended for a further 20 min. As a control for nonspecific binding to the beads, translated wild-type (WT) DP-1 was incubated with beads in the absence of p53. Beads were collected and washed three times (5 min each) in PI buffer minus BSA. SDS sample buffer was added to release bound protein. Depending on the size of the translated protein, samples were run on a 10, 12.5, or 15% gel. Assaying for competition between p53 and E2F-1 for binding to DP-1 in vitro was carried out essentially as described above, p53 being incubated with translated WT DP-1 together with an increasing amount of GST E2F-1 or GST alone.

Binding assay for DP-1. GST–DP-1 encodes a complete DP-1 protein fused to GST in pGEX-3X and was induced and purified by conventional procedures. E2F-1, E2F-4, and WT p53 were transcribed and translated in the presence [^{35}S]methionine in a TNT coupled lysate (Promega) as recommended by the manufacturer. For the in vitro binding assay shown in Fig. 5, fusion protein was added to the translate in PBSA (PBS containing 1 mM EDTA, 1 mM dithiothreitol, and 0.5% Tween 20) and incubated for 30 min at 30°C. Glutathione beads were subsequently added, and the mixture was incubated for a further 30 min. Beads were collected and washed four times in the same buffer before being solubilized in SDS sample buffer. To map the region in p53 to which DP-1 binds, a panel of p53 mutants was made. GST–p53 1-73, 1-143, and 1-235 were made by PCR using human p53 (php53Cl [47]) as a template. PCR products were cloned in frame into a pGEX vector (Pharmacia). Fusion proteins were induced and purified by conventional procedures. For the in vitro binding reaction shown in Fig. 6, approximately 10 μg of GST or GST-p53 fusion protein bound to glutathione-agarose beads was added to 15 μl of in vitro-translated DP-1 in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 10 mg of lysozyme per ml, 0.5 mM phenylmethylsulfonyl fluoride, 50 μg of leupeptin per ml, 50 μg of protease inhibitor per ml, 50 μg of aprotinin per ml, 50 mM dithiothreitol). After incubation for 2.5 h at 4°C, the beads were collected and washed four times in lysis buffer. Proteins were released in SDS sample buffer, electrophoresed, and immunoblotted with anti-DP-1(A) or anti-DP-1(D).

Transient transfection. The reporter constructs pDHFR-luciferase, pCMV-βgal, pCMV-E2F-1, and pCMV-DP-1 have been described previously (13, 24). pC53-SN3 encodes WT p53 driven by the cytomegalovirus enhancer/promoter region (1). The total amount of DNA in each transfection was made up with empty vector. Cells were transfected by the conventional calcium phosphate procedure. Luciferase and β-galactosidase assays were performed as described previously (13). Each treatment was performed in duplicate.

RESULTS

Distinct forms of DP-1 in vivo. Two distinct DP-1 polypeptides of 55-kDa, referred to as p55L (lower) and p55U (upper), can be resolved during cell cycle progression in 3T3 cells. p55L appears toward the end of G_1 as cells begin to enter S phase (4). To characterize the two forms of p55 in greater detail, we prepared antisera, monoclonal antibody 32.3 and polyclonal anti-DP-1(D), which recognize p55L. Upon immunoblotting of extracts prepared from asynchronous cultures of cells, a polyclonal antiserum, anti-DP-1(A), revealed both forms of p55 (Fig. 1a, track 1) (4), in contrast to monoclonal antibody 32.3, which defined p55L (Fig. 1a; compare tracks 1 and 2). Further evidence that 32.3 recognizes p55L was obtained upon immunoprecipitation with 32.3 in the presence and absence of the homologous peptide and subsequent probing of the immunoprecipitate with polyclonal anti-DP-1(A). The immunoprecipitated polypeptide comigrated with p55L (Fig. 1b; compare tracks 2 and 3).

The effect of 32.3 on DRTF1/E2F DNA binding activity was examined. In extracts prepared from F9 EC cells, the addition of 32.3 to the binding reaction caused an almost complete shift of DRTF1/E2F compared with the reaction performed in the presence of the homologous peptide (Fig. 1c; compare tracks 1 and 2); similar results were observed for extracts prepared from a wide variety of other types of cells (data not shown). Furthermore, 32.3 immunoprecipitated DRTF1/E2F DNA binding activity from F9 EC cell extracts (Fig. 1c; compare tracks 3 and 4), conditions in which p55L was the predominant form of DP-1 present in the immunoprecipitate (Fig. 1b, track 2). These data therefore argue that p55L is a significant component of DRTF1/E2F DNA binding activity.

To substantiate this idea, we studied the chromatographic

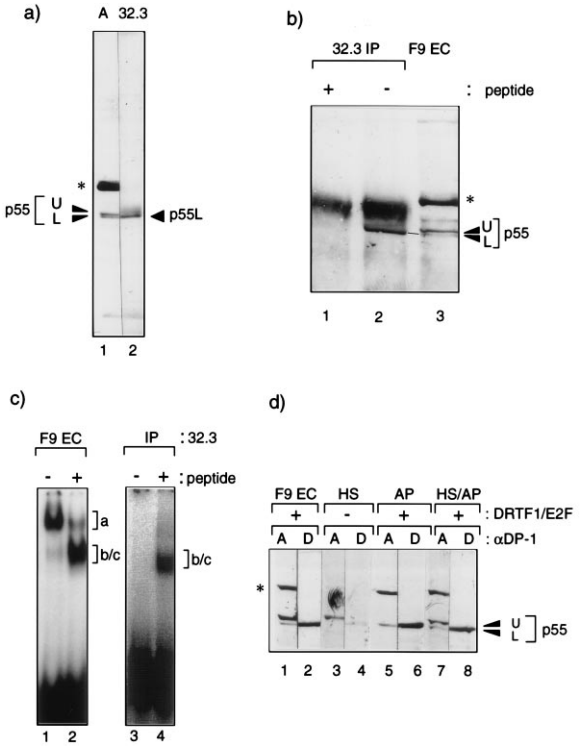

FIG. 1. Different forms of the DP-1 protein. (a) Immunoblotting was performed on extracts prepared from F9 EC cells with either polyclonal anti-DP-1(A) (track 1) or monoclonal antibody 32.3 (track 2). p55U and p55L are indicated, and the asterisk indicates an unrelated polypeptide defined by anti-DP-1(A) (4). Monoclonal antibody 32.3 is specific for the C-terminal D peptide, whereas anti-DP-1(A) is specific for the N-terminal A peptide of DP-1 (15). The track shown was cut in half along the middle, probed with each antiserum, and realigned after the reaction was complete. (b) Immunoprecipitation (IP) with monoclonal antibody 32.3 from F9 EC cell extracts was performed in the presence of the homologous (track 1) or an unrelated (track 2) peptide. The immunoprecipitate was subsequently immunoblotted with polyclonal anti-DP-1(A). A sample of the F9 EC extract was immunoblotted in parallel (track 3). p55U and p55L are indicated, and the asterisk indicates an unrelated polypeptide defined by anti-DP-1(A) (4). Note that the DP-1 polypeptide immunoprecipitated by 32.3 is p55L. The common polypeptide recognized in tracks 1 and 2 is caused by a nonspecific reaction with immunoglobulin released from the immunoprecipitation step. (c) DRTF1/E2F DNA binding activity was resolved in F9 EC cell extracts (tracks 1 and 2) in the presence of monoclonal antibody 32.3 together with either an unrelated (track 1) or the homologous (track 2) peptide. In tracks 3 and 4, 32.3 was used to immunoprecipitate DP-1 from F9 EC cell extracts (exactly as for panel b). Homologous peptide was then added to release immunoprecipitated protein, the DNA binding activity of which was further resolved by gel retardation. (d) Fractions derived from F9 EC cell extracts by fractionation on heparin-Sepharose (HS; tracks 3 and 4) or E2F binding site affinity chromatography (AP; tracks 5 and 6) or a mixture of both fractions (HS/AP; tracks 7 and 8) were immunoblotted with an anti-DP-1 antibody (αDP-1), either polyclonal anti-DP-1(A) (A; tracks 1, 3, 5, and 7) or anti-DP-1(D) (D; tracks 2, 4, 6, and 8). In parallel, DRTF1/E2F DNA binding activity in each fraction was assessed by gel retardation; + and − indicate the presence and absence of DNA binding activity (data not shown). Unfractionated extract was immunoblotted in tracks 1 and 2, and representative fractions from HS or AP chromatography were assessed in tracks 3 to 6. Each track was cut in half along the middle, probed with either antiserum, and realigned after the reaction was complete. p55U and p55L are indicated, and the asterisk indicates an unrelated polypeptide. Anti-DP-1(D) is a polyclonal rabbit antiserum which possesses the same specificity as monoclonal antibody 32.3.

properties of p55U and p55L during the fractionation of F9 EC cell extracts and correlated the presence of each polypeptide with the presence of DRTF1/E2F DNA binding activity within each fraction. For this analysis we used two antisera, anti-DP-1(A), which defines p55U and p55L, and anti-DP-1(D), which possesses the same specificity as monoclonal antibody 32.3 in recognizing p55L (Fig. 1d and data not shown). Extracts prepared from F9 EC cells were fractionated over heparin-Sepharose and subsequently assayed for DNA binding activity. Both p55U and p55L were present in the F9 EC cell extract (Fig. 1d, tracks 1 and 2), although after passage over heparin-Sepharose, p55L correlated with DRTF1/E2F DNA binding activity and p55U correlated with fractions which lacked DNA binding activity (Fig. 1d; compare tracks 1 and 2 with tracks 3 and 4). Further purification of DRTF1/E2F DNA binding activity by E2F binding site affinity chromatography (14) correlated with p55L (Fig. 1d; compare tracks 5 and 6), and when fractions containing p55L and p55U (with and without DRTF1/E2F DNA binding activity) were mixed together, the original composition of p55U and p55L in the unfractionated cell extract was reconstituted (Fig. 1d; compare tracks 7 and 1). Overall, these data argue that p55L is a significant component of DRTF1/E2F DNA binding activity and, further, that p55U is likely a form of DP-1 which binds less efficiently to the E2F site.

DP-1 associates with p53. A number of polypeptides with distinct molecular weights were detected when different polyclonal anti-DP-1 peptide antisera were used to sequentially immunoprecipitate DP-1 from radiolabeled extracts prepared from F9 EC cells (data not shown). To characterize these polypeptides in greater detail, we assessed whether antisera directed against previously identified polypeptides recognized them. DP-1 was present in immunoprecipitates obtained with the anti-p53 monoclonal antibody 421 (Fig. 2a; compare tracks 3 and 4). However, p55U rather than p55L preferentially coimmunoprecipitated with p53 (compare tracks 2 and 3).

To substantiate this result, additional evidence for an interaction between p53 and DP-1 was obtained from an assay in which a p53 fusion protein was incubated in an F9 EC cell extract. In these conditions, p53 specifically associated with DP-1 (Fig. 2b, tracks 1 to 4). As expected, a pRb fusion protein known to bind to DRTF1/E2F in F9 EC cell extracts (2) could interact with DP-1 (tracks 7 and 8). Of course, the data do not address whether the interaction is direct or indirect but nev-

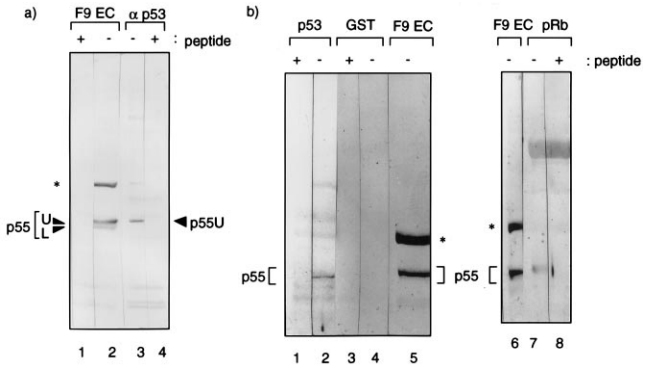

FIG. 2. p53 associates with DP-1. (a) Immunoprecipitation with the anti-p53 monoclonal antibody 421 from F9 EC cell extracts was performed as described in the text. The immunoprecipitate was subsequently immunoblotted with polyclonal anti-DP-1(A) in the presence of homologous peptide A (track 4) or an unrelated peptide (track 3). A sample of the F9 EC cell extract was immunoblotted with anti-DP-1(A) in parallel in the presence of competing peptides (tracks 1 and 2). p55U and p55L are indicated, and the asterisk indicates an unrelated polypeptide. (b) p53 (tracks 1 and 2), control GST protein (tracks 3 and 4), and pRb (tracks 7 and 8) fusion proteins were incubated in F9 EC cell extracts; bound proteins were eluted and subsequently immunoblotted with anti-DP-1(A) in the presence of the homologous peptide A (tracks 1, 3, and 8) or an unrelated peptide (tracks 2, 4, and 7). For comparison, an immunoblot of the F9 EC cell extract (10% of total input) is shown in tracks 5 and 6. p55 is indicated; GST-pRb served as a positive control.

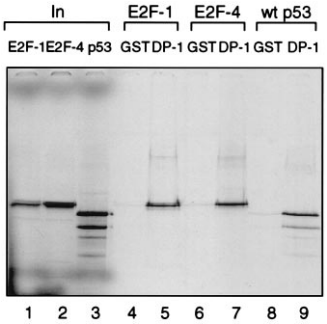

FIG. 3. p53 binds to DP-1 in vitro. GST–DP-1 (tracks 5, 7, and 9) or control GST protein (tracks 4, 6, and 8) was incubated with in vitro-translated E2F-1 (tracks 4 and 5), E2F-4 (tracks 6 and 7), or p53 (tracks 8 and 9), and bound proteins were resolved by SDS-gel electrophoresis. Tracks 1 to 3 show the input (In) in vitro-translated E2F-1, E2F-4, and p53.

ertheless do indicate that the association occurs in physiological conditions.

Mutational analysis of DP-1. To determine if DP-1 and p53 can interact in vitro and to compare the efficiency of interaction with the binding of DP-1 to E2F family members, we used an assay in which the ability of DP-1, expressed as a GST fusion protein, to bind to in vitro-translated p53 and E2F proteins was assessed. As expected, DP-1 could associate with either E2F-1 or E2F-4 in the conditions of this assay (Fig. 3; compare tracks 4 and 6 with tracks 5 and 7). Likewise, DP-1 could interact with p53 (compare tracks 8 and 9); similar results were obtained with the other members of the DP family, DP-2 and DP-3 (data not shown). The interaction between DP-1 and p53 was as efficient as the interaction between DP-1 and E2F-1 or E2F-4 and was specific, since p53 failed to bind to a control GST protein (tracks 4, 6, and 8). These in vitro binding data support the conclusion from the immunoprecipitation studies on the specific interaction of DP-1 with p53 in mammalian cells.

Using a similar assay, we determined the region in DP-1 required to bind to p53. A panel of mutant proteins derived from DP-1 representing N- and C-terminal truncations, together with a DP-1 protein altered at residues 172 and 173, were studied (Fig. 4e). In vitro-translated WT DP-1 bound to p53 (Fig. 4a; compare tracks 4 and 5), about 20% of the input DP-1 being retained by the WT p53 fusion protein. This binding efficiency was not significantly affected by removing up to 171 amino acid residues from the N-terminal region of DP-1 (Fig. 4d; compare tracks 1 with 2 and tracks 3 with 4), removing up to 79 amino acid residues from the C-terminal region (Fig. 4a [compare tracks 8 and 9] and Fig. 4d [compare tracks 1 with 2 and tracks 3 with 4]), or mutating residues 172 and 173 (Fig. 4a; compare tracks 2 and 3). However, further N-terminal deletion from residues 171 to 205 (Fig. 4c; compare tracks 7 and 8) or C-terminal deletion from residues 331 to 238 (Fig. 4c; compare tracks 1 and 2) significantly reduced the binding activity of DP-1 with p53. These data indicate that the minimal region in DP-1 capable of efficiently binding to p53 occurs within residues 171 to 331 (summarized in Fig. 4e). This region of DP-1 contains several domains which are conserved between other members of the DP family, notably DCB1 and DCB2 (13, 37), together with the DEF box, a critical region involved in heterodimerization between DP-1 and E2F family members (data not shown). Importantly, in the conditions of this assay in vitro-translated E2F-1 failed to interact with p53 (Fig. 4b; compare tracks 1 and 2), since the amount of E2F-1 retained by p53 was at background level (Fig. 4a and b; compare tracks 1 and 2). In the context of these assays, the DP protein appear to be the principal component which is capable of interacting with p53.

An immunochemically distinct form of DP-1 associates with p53. To define the region in p53 required for the association with DP-1, we developed a similar in vitro binding assay in which the ability of p53 to interact with in vitro-translated DP-1 was monitored. The data presented earlier indicated that p53 coprecipitates with p55U from mammalian cell extracts (Fig. 2a) and, further, that p55U is a form of DP-1 recognized by anti-DP-1(A) but not anti-DP-1(D) (Fig. 1d). In a similar fashion, two immunochemically distinct forms of DP-1 could be defined after in vitro translation using these same two antisera. Specifically, in the absence of translated exogenous DP-1, anti-DP-1(A), but not anti-DP-1(D), recognized the endogenous DP-1 protein (Fig. 5a, tracks 4 and 3, respectively). After translation, both antisera recognized the in vitro-translated DP-1 protein, the exogenous polypeptide being resolved with marginally faster mobility (tracks 1 and 2).

Evidence that at least two immunochemically distinct forms of DP-1 were present after in vitro translation was obtained upon studying the interaction with p53. When p53 was added to the in vitro translation mixture, the DP-1 form recognized by anti-DP-1(A), but not anti-DP-1(D), was retained by p53 (Fig. 5b; compare tracks 3 and 4) although DP-1 immunoreactive with both antisera was present in the input translation mixture (compare tracks 5 and 6); the GST portion failed to interact with DP-1 (tracks 1 and 2). These data indicate that two distinct forms of DP-1 are present after in vitro translation and further that p53 preferentially interacts with the form defined by anti-DP-1(A). Importantly, this result reflects the data derived by immunoprecipitation from mammalian cell extracts, in which case p53 coimmunoprecipitated with p55U, a DP-1 protein recognized by anti-DP-1(A) but not anti-DP-1(D). The specificity of p53 for DP-1 in the in vitro binding assay therefore possesses some similarity with the interaction in mammalian cells and supports the conclusion that p53 interacts with an immunochemically distinct form of DP-1.

An N-terminal region in p53 is required for binding to DP-1. We used the interaction of p53 and DP-1 to define the domain in p53 required for the association. As much as 250 amino acid residues could be deleted from the C terminus of p53 without any detrimental effect on the interaction with DP-1 (Fig. 6a and b; compare tracks 3, 4, and 5). A further deletion from residues 143 to 73 abolished the interaction (Fig. 6a and b; compare tracks 2 and 3), thus defining a region in p53 required to bind DP-1 (summarized in Fig. 6a). Since the N-terminal region of p53 contains the MDM2 binding domain (39), a domain in p53 necessary for the interaction with DP-1 can therefore be distinguished from the MDM2 binding domain.

p53 can modulate E2F site-dependent transcription. As DP-1 is a frequent component of DRTF1/E2F (3, 4), we assessed the functional consequence of the interaction of p53 with DP-1 by studying the effects on E2F site-dependent transcription driven by DP-1 and E2F-1, a situation in which it is known that both proteins cooperate in transcriptional activation as a DNA-binding heterodimer (Fig. 7, bars 1 to 6) (3). In these assay conditions, DP-1 alone possesses insignificant transcriptional activity (bars 1 to 16). When a WT p53 expression vector was cotransfected into 3T3 cells, the level of transactivation mediated by either E2F-1 alone or DP-1 together with E2F-1 was compromised in a p53 concentration-dependent fashion (compare bars 6 through 10 and bars 11 through 15). This inactivating effect of WT p53 was apparent in human SAOS-2 cells which contain a mutant p53 allele (Fig. 7). The activity of a comparable promoter construct driven by mutant

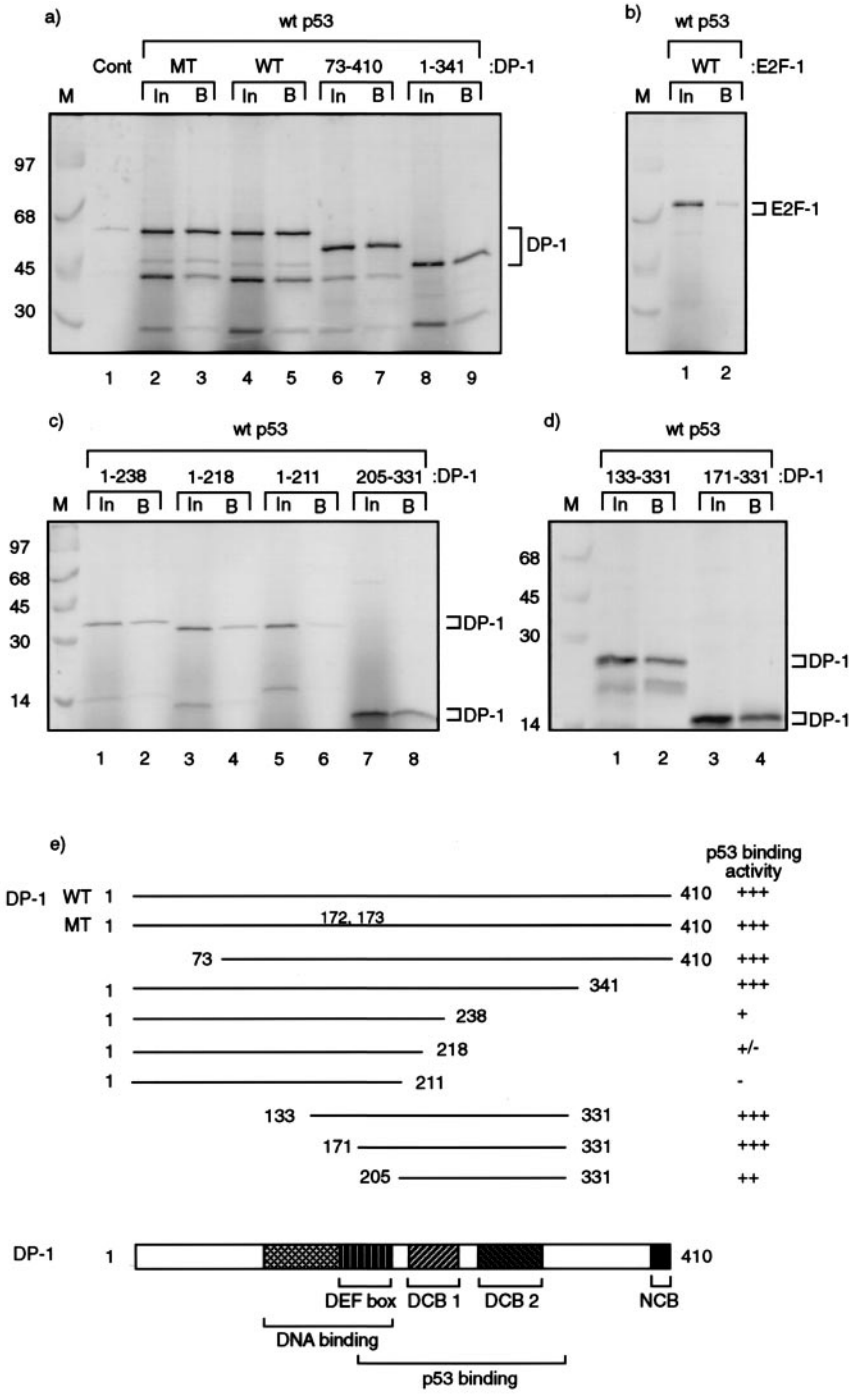

FIG. 4. Domains in DP-1 required for the interaction with p53. A p53 fusion protein (pH6-mmp53; see Materials and Methods) was used in the in vitro binding assay together with a panel of mutant DP-1 proteins (e). Each pair of tracks shows the input in vitro translation product on the left (In; 20% of total input) and the amount bound to the p53 fusion protein on the right (B). The details of the DP-1 mutant are indicated above each pair of tracks. Lane M contains molecular weight markers, the positions of which (in thousands) are shown on the left; the control binding activity with the beads is shown in panel a, track 1. In panel b, the binding activity of E2F-1 with p53 is indicated. The data are summarized in panel e; the region in DP-1 necessary for the efficient interaction with p53 and conserved domains in DP-1 are indicated. NCB, DCB1, DCB2, and the DEF box indicate conserved domains (13).

E2F binding sites, p3xMT-GL, was not significantly affected by p53 (data not shown). Furthermore, since this inactivating effect of p53 is calculated as a ratio of the reporter to the transcriptional activity of an internal control (pCMV-βgal) within the same transfection treatment, it is a specific effect of transcriptional activation through the E2F site.

p53 and E2F-1 compete for DP-1. It was of interest to determine if the interaction of p53 with DP-1 was mutually exclusive with the interaction of DP-1 and E2F-1. To address this question, we assessed whether E2F-1, expressed as a GST fusion protein, would compete with p53 for DP-1 in the in vitro binding assay, conditions in which in vitro-translated DP-1

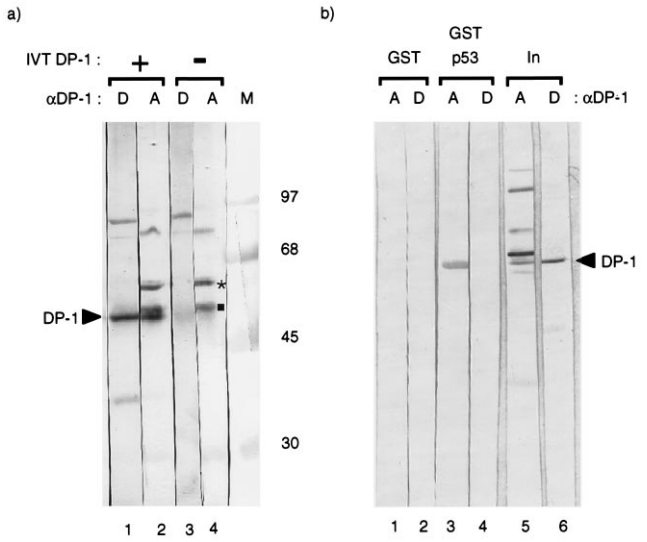

FIG. 5. p53 binds to an immunochemically distinct form of DP-1. (a) Reticulocyte lysate programmed either with (tracks 1 and 2) or without (tracks 3 and 4) DP-1 was probed with an anti-DP-1 (αDP-1) antibody, either anti-DP-1(D) (D; tracks 1 and 3) or anti-DP-1(A) (A; tracks 2 and 4). The in vitro-translated (IVT) DP-1 polypeptide is indicated by the arrowhead; note that the antisera were affinity purified. The polypeptide indicated by the box and recognized by anti-DP-1(A) in track 4 is probably endogenous DP-1; the asterisk indicates a nonspecific polypeptide. Lane M contains molecular weight markers, the positions of which (in thousands) are indicated on the right. (b) Either GST-p53 (tracks 3 and 4) or GST alone (tracks 1 and 2) was incubated with in vitro-translated WT DP-1, and the bound DP-1 was assessed by immunoblotting with either anti-DP-1(A) (tracks 1 and 3) or anti-DP-1(D) (tracks 2 and 4). Each track was cut in half along the middle, probed with either antiserum, and realigned. In tracks 5 and 6, the input (In) reticulocyte lysate containing in vitro-translated DP-1 was probed with anti-DP-1(A) and anti-DP-1(D), respectively; DP-1 is indicated by the arrowhead. Note that GST-p53 binds a form of DP-1 recognized by anti-DP-1(A) but not anti-DP-1(D).

binds to p53 (Fig. 4a, compare tracks 1 and 9). As the amount of GST-E2F-1 was increased, there was a concomitant reduction in the level of DP-1 bound to p53, an effect not apparent in the control GST treatment (Fig. 8a; compare tracks 2 to 5 with tracks 7 to 10). These data indicate that p53 and E2F-1 compete for binding to DP-1 and are consistent with the earlier data indicating that p53 interacts with the dimerization domain of DP-1.

If p53 and E2F-1 compete for DP-1, reduced DNA binding activity due to the DP-1/E2F-1 heterodimer may be apparent in the presence of p53. To test this possibility, a band shift assay which measured the DNA binding activity of the DP-1/E2F-1 heterodimer was supplemented with p53. After in vitro translation, DP-1 or E2F-1 alone had little DNA binding activity, although when assessed together, they were found to cooperate (Fig. 8b; compare tracks 2, 3, and 5). As the level of p53 in the reaction mixture increased, reduced DP-1/E2F-1 DNA binding activity was apparent (compare tracks 5, 6, 8, and 10). In contrast, inactivated p53 had little effect (compare tracks 7, 9, and 11). In conclusion, p53 and E2F-1 compete for DP-1, and as a result, p53 can reduce the level of DNA binding activity of the DP-1/E2F-1 heterodimer.

DISCUSSION

p53 associates with DP-1. In this report, we have described the association between DP-1 and p53 in mammalian cells. The region in p53 required for the interaction with DP-1 exists within the N-terminal 143 amino acid residues. The first 73 residues, which contain the MDM2 binding domain (39), are not sufficient for the interaction. Although previous studies have suggested that MDM2 can interact with DRTF1/E2F (31), our results imply that this interaction is unlikely to be responsible for the association of DP-1 with p53. Interestingly, the region between residues 73 and 143, which is necessary for p53 to bind DP-1, contains residues frequently altered in human tumor cells carrying mutant p53 alleles (16).

Although p53 can associate with DP-1, we have not been able to detect p53 in physiological DRTF1/E2F DNA binding complexes (data not shown). This is consistent with the interaction of p53 with p55U, but not p55L, since p55U is a rare component of physiological DRTF1/E2F DNA binding activity. The results derived from the in vitro assay in which p53 specially binds to DP-1 support these observations, since they also indicate that p53 binds to an immunochemically distinct form of DP-1. Overall, these data suggest that p53 targets a subpopulation of DP-1 and, further, that this form of DP-1 is an infrequent component of DRTF1/E2F DNA binding activity. Since it is known that DP-1 undergoes regulated phosphorylation (4, 27), it is possible that p53 targets a particular posttranslational form of DP-1, although further studies are required to clarify this possibility.

Coexpression of p53 specifically inactivated transcription driven by the DP-1/E2F-1 heterodimer. Given the earlier conclusion, a potential model to explain these results would be

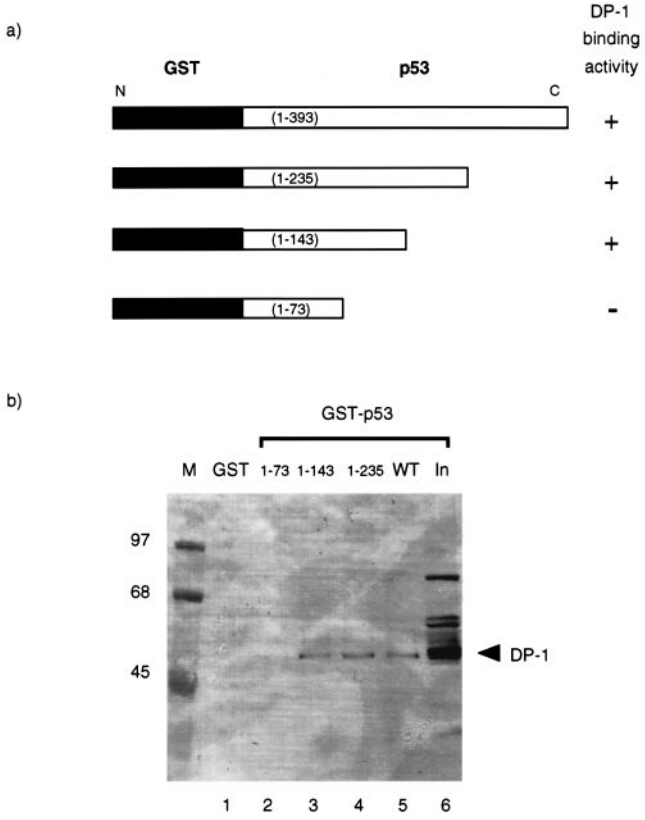

FIG. 6. Domains in p53 required for the interaction with DP-1. (a) A panel of GST-p53 fusion proteins were used in an in vitro binding assay with DP-1; bound in vitro-translated DP-1 was detected by immunoblotting with anti-DP-1(A). (b) The indicated p53 fusion proteins (tracks 2 to 5) or control GST (track 1) was incubated with in vitro-translated WT DP-1 (track 6), and the bound DP-1 was assessed by immunoblotting with anti-DP-1(A). Lane M contains molecular weight markers, the positions of which (in thousands) are indicated on the left. In, input.

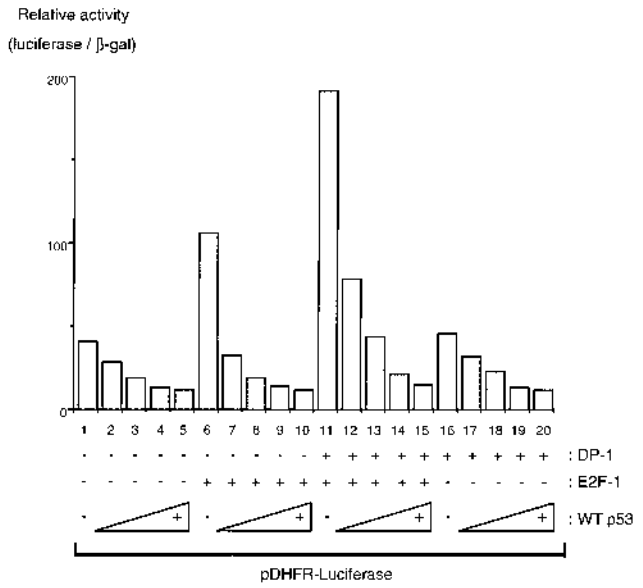

FIG. 7. p53 modulates E2F binding site-dependent transcription. SAOS-2 cells were transfected with expression vectors encoding E2F-1 (bars 6 to 15; 200 ng) and DP-1 (bars 11 to 20; 2 μg), together with an increasing amount of the p53 expression vector (bars 2, 7, 12, and 17, 1.0 μg; bars 3, 8, 13, and 18, 2.5 μg; bars 4, 9, 14, and 19, 7.5 μg; bars 5, 10, 15, and 20, 15.0 μg). The reporter construct was pDHFR-luciferase, containing positions −270 to +20 from the *DHFR* promoter, and pCMV-βgal was included in each transfection as an internal control; the values shown are based on the activity of pDHFR-luciferase (luciferase) relative to that of pCMV-βgal (β-gal). The p53 expression vector suppressed the activity of pCMV-βgal, which was taken into consideration in determining the effect of p53 on DP-1 and E2F-1. The values shown represent the means of two readings.

that p53 holds DP-1 in a state which prevents it interacting with an E2F family member to form a DP-1/E2F heterodimer. Indeed, the region in DP-1 required to interact with p53 is necessary to form a DP-1/E2F-1 heterodimer (3, 18, 28), and thus binding of p53 to DP-1 could be mutually exclusive with the interaction of DP-1 with E2F family members. Evidence for such a possibility was obtained by demonstrating that p53 and E2F-1 can compete for DP-1 and, consequently, reduce the level of DP-1/E2F-1 DNA binding activity. These data are compatible with a model in which p53 targets an immunochemically distinct form of DP-1, regulating the formation of DP-1/E2F heterodimers and hence the level of DRTF1/E2F DNA binding activity.

The region in p53 necessary for the interaction with DP-1 includes residues frequently altered in naturally occurring mutant alleles (16). On the basis of the results reported here, a potential biological rationale for these mutations is that they prevent p53 from interacting with DP-1 and thus relieve the negative regulation imposed by p53 on the formation of functional DRTF1/E2F and hence cell cycle progression.

A pathway for p53-mediated growth arrest. Although p53 is believed to possess the properties of a transcription factor and the transactivation of target genes, such as *gadd45* and *WAF1* (12, 26), is thought to be important in p53-mediated growth arrest, the interaction of p53 with DP-1 provides another potential pathway through which p53 may influence cell cycle progression. Thus, since many of the genes regulated by DRTF1/E2F encode proteins required for cell cycle progression, their transcriptional down-regulation through the interaction of p53 with DP-1 may impede cell cycle progression.

Indeed, a variety of previous studies already have suggested that the pathways regulated by DRTF1/E2F and p53 are physiologically integrated. For example, overexpression of E2F-1 in cells induces apoptosis in a p53-dependent fashion (45), and the increased level of apoptosis in the lens fiber cells of $Rb^{-/-}$ mice is overcome in embryos which are doubly null in *Rb* and *p53* (33). Similar conclusions have been made from studies in which the oncoproteins of tumor viruses, which can inactivate pRb or p53, are sequentially targeted to defined physiological sites (21, 33, 38). Overall, such studies suggest that p53 monitors, in some as yet unknown way, the status of the DRTF1/E2F pathway. It is possible that the interaction between DRTF1/E2F and p53 is involved in regulating apoptosis, an idea which will be pursued in future studies.

Finally, the interaction of DP-1 and p53 may help explain the mechanism through which DP-1 exerts high levels of protooncogenic activity, a property which is shared by other members of the DP family and one manifest in the absence of a cotransfected E2F family member (24). It is possible the increased level of DP-1 sequesters p53, titrating out its activity and thus overriding the growth-regulating effects of p53. In these conditions, DP-1 may act analogously to certain viral oncoproteins, such as the adenovirus E1b and papillomavirus

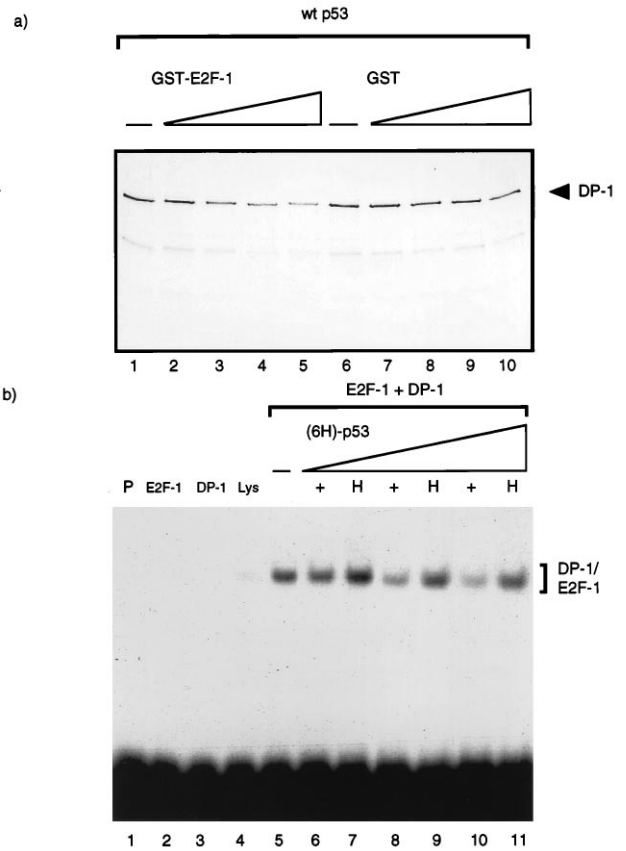

FIG. 8. p53 and E2F-1 compete for DP-1, and p53 inactivates DP-1/E2F-1 DNA binding activity. (a) About 50 ng of p53 fusion protein encoded by pH6-mmp53 was incubated with in vitro-translated DP-1 (100% of input shown in track 1) in the presence of an increasing amount of either GST-E2F-1 (tracks 2 to 5; ~25, ~50, ~125, and ~300 ng, respectively) or GST alone (tracks 7 to 10; ~25, ~50, ~125, and ~300 ng, respectively), and the amount of bound DP-1 was determined. The binding activity in the absence of either GST–E2F-1 or GST alone is shown in tracks 1 and 6. (b) Gel retardation assay was performed to assay the DNA binding activity of the DP-1/E2F-1 heterodimer (track 5) in the presence of an increasing amount of His_6-tagged p53 before (tracks 6, 8, and 10) or after (tracks 7, 9, and 11) denaturation. The amounts of p53 added were 15 ng (tracks 6 and 7), 25 ng (tracks 8 and 9), and 35 ng (tracks 10 and 11). The activities of E2F-1, DP-1, and the reticulocyte lysate (Lys) are indicated (tracks 2 to 4); and the probe (P) alone is shown in track 1. (6H)-p53, His_6-tagged p53.

E6 proteins, since their ability to inactivate p53 also correlates with oncogenic activity (34).

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The first three authors contributed equally to this work.

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